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Disseminated Intravascular Coagulation in Macaca mulatta
with Experimental Bolivian Hemorrhagic Fever

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Running head: Disseminated Intravascular Coagulation

In conducting the research described in this report, the investigators
adhered to the "Guide for the Care and Use of Laboratory Animals," as
promulgated by the Committee on the Revision of the Guide for Laboratory
Animal Facilities and Care of the Institute of Laboratory Animal
Resources, National Research Council. USAMRIID facilities are fully
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Department of the Army or the Department of Defense.

The authors acknowledge the valuable technical assistance given
by Mr. George A. Fry and Mr. William G. Mahlandt.

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Disseminated Intravascular Coagulation in <u>Macaca mulatta</u> with Experimental Bolivian Hemorrhagic Fever		5. TYPE OF REPORT & PERIOD COVERED Interim
6. AUTHOR(s) S. K. Scott, R. L. Hickman, C. M. Lang, G. A. Eddy, D. E. Hilmas; and R. O. Spertzel		6. PERFORMING ORG. REPORT NUMBER
7. PERFORMING ORGANIZATION NAME AND ADDRESS U.S. Army Medical Research Institute of Infectious Diseases SGRD-UIA-A Fort Detrick, Frederick, Maryland 21701		8. CONTRACT OR GRANT NUMBER(s)
9. CONTROLLING OFFICE NAME AND ADDRESS		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62776A 3M762776A841 00 017
11. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE 10 Jun 77
		13. NUMBER OF PAGES 24
		14. SECURITY CLASS. (of this report) UNCLASSIFIED
15. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report) B		
17. SUPPLEMENTARY NOTES Reprints bearing assigned AD number will be forwarded upon receipt. To be submitted for publication in the <u>Journal of Infectious Diseases</u> .		
18. KEY WORDS (Continue on reverse side if necessary and identify by block number) Bolivian hemorrhagic fever Experimental infection Monkeys Disseminated intravascular coagulation		
19. ABSTRACT (Continue on reverse side if necessary and identify by block number) Experimental infection of rhesus monkeys (<u>Macaca mulatta</u>) with Machupo virus produced a hemorrhagic disease similar to that of Bolivian hemorrhagic fever (BHF) in humans. The disease in infected animals was also characterized by the development of hypotension and disseminated intravascular coagulation (DIC). The laboratory findings of severe thrombocytopenia, prolongation of the activated partial thromboplastin time (APTT), and increased concentrations of serum fibrin split products in six of the eight infected monkeys were used to make a diagnosis of DIC. The prolongation of the APTT was indicative of an (cont'd)		

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ABSTRACT

Experimental infection of rhesus monkeys (Macaca mulatta) with Machupo virus produced a hemorrhagic disease similar to that of Bolivian hemorrhagic fever (BHF) in humans. The disease in infected animals was also characterized by the development of hypotension and disseminated intravascular coagulation (DIC). The laboratory findings of severe thrombocytopenia, prolongation of the activated partial thromboplastin time (APTT), and increased concentrations of serum fibrin split products in six of the eight infected monkeys were used to make a diagnosis of DIC. The prolongation of the APTT was indicative of an abnormality in the intrinsic blood coagulation system, and was interpreted as indirect evidence of Hageman factor activation, which was the prime mediator of the DIC and hypotension observed.

Disseminated intravascular coagulation (DIC) is a clinical disorder which has been diagnosed in a wide variety of diseases, including those caused by bacterial and viral infections [1-5]. Clinically, DIC is expressed as a hemorrhagic diathesis resulting from the depletion of critical blood coagulation factors [3-5]. Other adverse effects of DIC can result from the obstruction of the microcirculation by the deposition of thrombi [6,7]. This can cause localized ischemia and necrosis, or a generalized circulatory collapse [2,6,7]. The most common clinical characteristics of DIC in humans are prolongation of the prothrombin time (PT), thrombocytopenia, and hypofibrinogenemia [4,5]. The diagnosis is usually made on the basis of these three abnormalities, or, in the event of inconclusive results, with the aid of additional tests such as activated partial thromboplastin time (APTT) and serum fibrin split products (FSP) [4,5].

There are several reports of this disorder, in both man and animals, resulting from bacterial infections [1,4,8]. The relationship of viral infections to DIC, however, is not as clearly understood. It has been suggested that DIC may be involved in the pathogenesis of viral hemorrhagic fevers such as Bolivian hemorrhagic fever (BHF), a disease caused by Machupo virus, a member of the arenaviridae group of viruses [3]. This disease is characterized clinically by high fever, severe headache, myalgia, cutaneous hyperesthesia, anorexia, vomiting, diarrhea, epigastric pain, and bleeding tendencies [9-12]. Near the end of the febrile phase, hypotension becomes clinically noticeable and often leads to shock and death [9]. During the development of hypotension, body temperature commonly decreases to near normal values or slightly below [9].

Clinical laboratory determinations in reported cases are limited; however, it is known that the illness is characterized by leukopenia, thrombocytopenia, proteinuria, and increased packed cell volume during the febrile phase [9-12]. Both CF and serum-neutralizing antibodies to the virus can be detected from 25 to 30 days after illness [9]. Consistent histopathologic lesions in fatal human disease include hepatic necrosis, interstitial pneumonia, and congestion and hemorrhage in various tissues [13].

Although many of the clinical and pathologic lesions of BHF in humans indicate the presence of DIC, its pathogenic mechanism is not clearly understood. The rhesus monkey (Macaca mulatta) has been used as a model for experimental Machupo virus infection to study both clinical signs and pathologic lesions in BHF [14-16]. Many of the findings in monkeys closely resemble those that have been reported in humans severely ill with the disease. It was the purpose of this study to determine the role of intravascular coagulation in the pathogenesis of BHF in M. mulatta experimentally infected with Machupo virus.

Materials and Methods

Animals. Eighteen healthy, young, adult M. mulatta were used. Monkeys were chosen without regard to sex, and ranged in weight from 2.5 - 5.0 kg. They were randomly divided into two groups. Group 1 consisted of six virus-infected and three noninfected monkeys which were used for arterial blood pressure and heart rate measurements. Group 2 was composed of eight virus-infected and four noninfected monkeys which were used in blood coagulation studies. Group 1

noninfected controls were reused in group 2.

Animals used in the blood coagulation studies were housed in galvanized steel cages within a biological containment facility, previously described [17]. All of the monkeys were provided water and standard monkey chow ad libitum.

Sterile intravenous and intraarterial catheters were surgically implanted in the monkeys under Fluothane^R (Ayerst Laboratories, New York, N.Y.) anesthesia by the method of Wakeley et al. [18] with the following modifications. Arterial blood pressure and heart rate were determined using a 19-gauge firm polyethylene catheter placed in the left carotid artery and directed into the dorsal aorta. A flexible 18-gauge Silastic catheter (Dow Corning Corp., Midland, Mich.) was placed in the left external jugular vein and directed into the inferior vena cava for the purpose of obtaining venous blood samples. Immediately following surgery, anesthetized monkeys were placed in primate restraining chairs in the biological containment facility. Patency of the catheters was maintained by flushing with heparinized 0.9% saline.

Rectal temperatures were measured at the same time each day. A commercially available Hemocult^R kit (Smith, Kline Diagnostics, Sunnyvale, Cal.) was used to test for occult blood in the feces of each monkey once daily.

All virus-infected monkeys died and were necropsied. Tissues were fixed in 10% neutral phosphate buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for examination by light microscopy.

Physiological measurements. Arterial blood pressure and heart

rate were recorded in chair-restrained monkeys by the method of Forsyth et al. [19]. The carotid artery catheters were connected to P23 Statham strain gauge pressure transducers (Gould, Inc., Cleveland, Ohio) with leads attached to a Model 200 Brush recorder (Gould, Inc.). Recordings were made at the same time each day from each monkey for approximately 5 min. Heart rate (beats/min), diastolic and systolic pressures (mm Hg) were obtained directly from the recordings. Pulse pressure was calculated by subtracting diastolic from systolic pressure. Mean pressure (mm Hg) was calculated as one-third of the pulse pressure plus diastolic pressure. Recordings were made on five consecutive days to establish base-line values.

Hematologic determinations. A venous blood sample was obtained from each monkey on three alternate days during a five-day pre-experimental period to determine base-line values for each variable studied. Immediately after the final base-line sample was obtained, the monkeys were inoculated sc with either 0.5 ml of 0.9% saline or 0.5 ml of a suspension of the Carvallo strain of Machupo virus appropriately diluted to contain 1×10^3 pfu of virus.

Postinoculation blood samples were also obtained on alternate days during the week; no samples were obtained on weekends. Blood samples were obtained for a period of three to four weeks, including the pre-experimental base-line period. Venous blood was decanted into sterile plastic tubes containing either EDTA, sodium citrate, or no additive. Blood specimens containing sodium citrate (one volume 3.8% sodium citrate to nine volumes of whole blood) were used for FSP, fibrinogen, PT, and APTT determinations. These specimens were centrifuged at 2,500 rpm for 20 min, after which the supernatant

plasma was pipetted into separate plastic tubes. For FSP and fibrinogen assays, 0.5 ml of plasma was added to 0.5 ml of a clotting solution (pH 7.3) containing 25 mM CaCl_2 , 308 mM NaCl, 100 mM ϵ -aminocaproic acid, and 25 mM Tris (Parke, Davis and Co., Detroit, Mich.). The plasma and clotting solution mixture was incubated at 22 C for 60 min, with gentle digital shaking every 10 - 15 min to aid in clot retraction. After incubation, the clot was compressed against the side of the tube with a small laboratory spatula. The clotted sample was then centrifuged at 4,500 rpm for 20 min. The supernatant serum was pipetted into a separate plastic tube and stored at -70 C until assayed for FSP. The clot remaining in the tube was stored at -70 C until assayed for fibrinogen concentration, which was by an automated Lowry technique [21] using an Auto Analyzer II^R system (Technicon Instrument Corp., Tarrytown, N.Y.).

Serum FSP were measured by Laurell's method of electroimmunoassay [20] with the following modifications. The fibrinogen-related antigen standards were dilutions of pooled rhesus monkey plasma containing 555 μg fibrinogen/ml. Commercially available rabbit antiserum against rhesus fibrinogen (Cappel Laboratories, Inc., Doughton, Pa.) was incorporated into the agar gel.

Simplastin^R (General Diagnostics, Morris Plains, N.J.) was used in determining PT and Automated APTT^R (General Diagnostics) for determining APTT, as recommended by the manufacturer. Clot formation was timed (seconds) automatically by the use of a fibrometer (Balt Biological Laboratories, Cockeysville, Md.) in both tests.

Blood samples mixed with EDTA (0.02 ml EDTA to 1.0 ml whole blood) were used immediately to determine thrombocyte counts and packed cell volume. Thrombocytes were counted in a hemacytometer after preparation

by the Unopette^R method (Becton, Dickinson and Co., Rutherford, N.J.). Packed red blood cell volumes were determined as described previously [15].

Serum was used for sorbitol dehydrogenase (SDH), albumin, electrolytes (Na^+ , K^+), viremia, and neutralizing antibody determinations. Serum to be assayed for SDH or albumin was held at 4 C until testing, which was within 1 hr after sample collection. Serum samples for the other variables mentioned were stored at -70 C until used.

Serum SDH was measured spectrophotometrically by the method of Weisner et al. [22] using a commercially available kit (Sigma Chemical Corp., St. Louis, Mo.). Albumin was also measured spectrophotometrically using a commercial kit (American Monitor Corp., Indianapolis, Ind.). Electrolyte analyses were done by flame photometry (Model #143, Instrumentation Laboratory, Lexington, Mass.).

Virus assays were performed by the direct enumeration of virus plaque on Vero cells (a continuous line of African green monkey kidney cells). Assays for neutralizing antibody were based on the highest dilution of serum that inhibited 80% or more of the Marupo virus plaques used. The virus, virus assay, and neutralizing antibody determinations have all been described previously [15,23].

Statistical analysis. Group mean values were compared by use of a "t" test statistic where appropriate.

Results

Clinical signs. The clinical signs of disease in the virus-infected monkeys closely resembled those in experimentally

infected rhesus monkeys previously described by Kastello et al. [15] and included: lethargy, decreased appetite (to complete anorexia), dehydration, epistaxis, skin petechiae, and bleeding gums. Occult fecal blood was an infrequent finding. Although the illness was inevitably fatal in infected monkeys, it was more acute in the chair-restrained than cage-confined animals, with death occurring at an average of 13 days postinoculation in the former and 21 days in the latter.

Rectal temperatures began to increase in the chair-restrained infected monkeys on day 3 postinoculation (figure 1). The mean level of fever exceeded 39.5 C. Temperatures began to decrease after day 7, and were less than 36 C in all infected monkeys for 12 - 24 hr before death.

Physiological measurements. Mean arterial blood pressure was similar in infected and noninfected chair-restrained monkeys until day 6 postinoculation (figure 1). Subsequent to day 6, mean blood pressure increased gradually in the noninfected controls until day 9 when the early preinoculation levels were again reached. By contrast, infected monkeys showed a decrease in mean blood pressure beginning on day 6 postinoculation. This decrease in blood pressure coincided roughly with defervescence in infected monkeys. Mean blood pressure of infected monkeys was significantly decreased ($p < 0.05$) on days 7 and 9 - 14 when compared to noninfected controls. However, critical hypotension (to less than 55 to 60 mm Hg) was not seen in any of the infected monkeys until the last two or three days of illness before death. There was no significant variation in heart rate between the infected and noninfected monkeys until the terminal stage of the

illness when heart rate decreased dramatically in infected monkeys which were in a moribund condition.

Hematologic determinations. Thrombocytopenia occurred in each of the cage-confined infected monkeys. Mean thrombocyte counts of the infected group were significantly lower ($P < 0.05$) than those of the noninfected group on days 5 - 21 post inoculation (figure 2).

There was no significant variation in the mean PT in the infected monkeys when compared to noninfected controls during the study. In contrast, prolongation of the APTT in infected monkeys was dramatic (figure 2). Onset of prolonged APTT coincided with defervescence at day 7 postinoculation. The APTT was significantly increased ($P < 0.05$) in infected monkeys when compared to the noninfected controls on days 7 - 21 (figure 2).

Serum FSP were detected in six of the eight infected monkeys from days 12 to 21 postinoculation (figure 2). The highest mean concentration of FSP in the infected group, 10.6 ug/ml serum, occurred on day 14 postinoculation. The levels of serum FSP in infected monkeys were significantly different ($P < 0.05$) on days 14 and 17, when compared to noninfected controls.

Mean serum SDH levels were elevated in infected monkeys as early as day 4 postinoculation, and were significantly greater ($P < 0.05$) than those of noninfected controls on days 7 - 21 (figure 3). The highest mean serum concentration in the infected group, 1,190 Sigma units/ml, was observed on day 17 postinoculation.

Serum albumin concentrations were significantly lower ($P < 0.05$) in infected monkeys when compared to noninfected controls on days 5 - 21 postinoculation (figure 3). Mean levels of serum albumin in

infected monkeys decreased almost 50% from preinoculation levels by day 7 postinoculation, and remained depressed throughout the illness. Changes in serum albumin concentration coincided with increased serum SDH levels in the infected monkeys (figure 3).

Mean plasma fibrinogen concentration increased in the infected monkeys beginning on day 7 postinoculation (table 1), and remained elevated in five throughout the illness. The levels were decreased in the remaining three monkeys from days 19 - 21.

Mean packed cell volumes decreased progressively in infected monkeys from day 3 postinoculation throughout the illness. Packed cell volumes were decreased by 40 - 45% of the preinoculation values in infected monkeys during the illness. There were no significant changes in the serum electrolyte (Na^+ , K^+) concentrations in the infected monkeys during the study.

Viremia and antibody responses. The geometric mean virus titers in sera of infected monkeys were similar to those previously described for rhesus monkeys by Kastelle et al. [15]. Four of six chair-restrained monkeys became viremic by day 5 postinoculation, and all of them were viremic by day 7. In contrast, four of eight cage-confined monkeys were viremic by day 5 postinoculation, and all were, by day 10. Viremia remained at high levels throughout the illness in all infected monkeys, and the majority developed no detectable serum neutralizing antibody to Machupo virus prior to death. Low level antibody titers of 1:16 were detected in only two monkeys on day 21 postinoculation.

Pathologic lesions. Typical gross pathologic lesions observed during necropsy of infected monkeys included pale yellow livers, skin

petechiae, and gingival hemorrhages. The most common microscopic lesions observed were hepatic necrosis and necrotic enterocolitis. Less frequent microscopic lesions included esophagitis, pharyngitis, cystitis, adenitis, pulmonary and myocardial hemorrhages and nonspecific degenerative changes in vascular endothelial cells. Fibrin thrombus deposition was detected in only one infected animal, in the renal glomerular capillaries.

Discussion

The experimental infection of M. mulatta with Machupo virus produced a hemorrhagic disease similar to that of BHF in humans, although the mortality rate was higher than that normally occurring in man [9]. The disease in monkeys was also characterized by DIC and hypotension.

Machupo virus infection in chair-restrained animals caused hypotension similar to that which has been reported in humans with the disease [9]. Decreases in mean blood pressure correlated with hypothermia, as had been seen in man [9]. Hypotension developed gradually and reached critical levels at the terminal stage of the illness in each animal.

The appearance of nonspecific degenerative changes in vascular endothelium during the acute phase of the disease is consistent with previous observations of others [16]. Eddy et al. [14] reported widespread vasculitis during the later, encephalitic phase of Machupo virus infection in M. mulatta. The nonspecific changes seen in the acute phase are probably the forerunner of the vasculitis reported in the encephalitic phase, and this injury could have induced thrombosis

factor (factor XII) activation. It has been suggested that viral infections can initiate DIC through the intrinsic blood coagulation pathway by endothelial injury which induces Hageman factor activation [4,6]. The pathogenesis of the hypotension that occurred in infected M. mulatta may also have been due to Hageman factor activation. Once activated, Hageman factor catalyzes the in vivo conversion of the inactive plasma enzyme kallikreinogen into its active form, kallikrein [24]. Kallikrein then cleaves plasma kininogen, producing the potent vasodilator, bradykinin, which, in turn, causes hypotension [4,24].

The finding of thrombocytopenia, prolonged APTT, and elevated serum FSP in six of the eight cage-confined infected monkeys was used for a diagnosis of DIC in these animals. Although the thrombocytopenia and prolonged APTT were significant in the remaining two monkeys, the results of the assays for serum FSP were inconclusive for a diagnosis of DIC.

Thrombocytopenia is the most consistent clinical abnormality found in human patients with DIC, regardless of the underlying disease precipitating the disorder [1,5]. Similarly, it was observed in all eight of the infected monkeys. In thrombocytopenia, red blood cells may be lost through blood vessel walls and subsequently enter the lymphatic system and appear finally as petechiae or ecchymoses of the skin [6]. This was commonly seen in the form of skin petechiae in the infected monkeys in this study.

The exact cause of thrombocytopenia in infected monkeys was not determined. It may have been the results of depletion of thrombocytes by intravascular coagulation, decreased production of thrombocytes due to viral-induced bone marrow depression, or both [6].

The severe prolongation of the APTT, which occurred in all eight of the infected monkeys, was indicative of a critical abnormality in the intrinsic coagulation pathway [6]. The APTT is especially sensitive to deficiencies of clotting factors V and VIII through XII; a deficiency of any one of these factors could result in prolonged APTT [6].

The extrinsic coagulation pathway was also considered, but did not seem to be involved in the pathogenesis of the coagulopathy in infected monkeys. Prolongation of the PT, which is indicative of an abnormality in the extrinsic coagulation pathway, was slight in infected monkeys and at no time was it determined to be clinically abnormal. Although the PT is less sensitive to clotting factor deficiencies than APTT [4], we concluded that the extrinsic coagulation system was not involved in the pathogenesis of the coagulopathy in the infected monkeys.

Although there was a critical deficiency in the intrinsic system in the infected monkeys, as indicated by the prolongation of the APTT, the occurrence of serum FSP in six of them substantiated a diagnosis of DIC. The release of excess FSP into the circulation as a result of enhanced fibrinolysis may exacerbate the hemorrhagic diathesis associated with DIC by inhibiting thrombocyte functions (adhesion and aggregation to subendothelial connective tissue) and fibrin monomer polymerization [6].

The concentration of fibrinogen in plasma of human patients with DIC may remain normal or become slightly elevated, due to an equilibrium between the rate of degradation of fibrin and the synthesis of fibrinogen [4,5]. It has been reported that the release of FSP

into the blood may signal the release of stored fibrinogen from the liver [25]. These phenomena are consistent with the elevation in plasma fibrinogen concentration in the virus-infected monkeys which was accompanied by the appearance of FSP in the blood.

Hepatocellular necrosis occurred in infected monkeys as early as day 4 postinoculation and became increasingly severe as the disease progressed, indicated by the progressive elevation in serum SDH concentrations. The elevated serum SDH concentrations correlated with hypoalbuminemia and prolongation of the APTT. This prolongation may have been due to deficiencies of blood coagulation factors I or V, which are synthesized by the liver, or II, IX, or X, all of which require vitamin K for synthesis by the liver [4]. The hypoalbuminemia was probably due to decreased liver synthesis because of severe hepatocellular necrosis, and may have caused or exacerbated the systemic arterial hypotension in infected monkeys by resulting in a decrease in the colloid osmotic pressure of plasma [26,27].

Packed cell volumes in the cage-confined infected monkeys may be affected by such factors as dehydration, red blood cell size, and the method of assay. A decrease in packed cell volume is difficult to explain in this study, as there was little evidence of hemolysis in serum samples and the bleeding tendencies noted clinically were not severe. Precise measurements of the state of hydration in the animals were not done; however, the infected animals did show clinical signs of dehydration such as decreased skin elasticity and sunken orbits. Therefore, decreased packed cell volumes occurred at a time when we expected the reverse to occur.

Tests for occult fecal blood were positive in only two infected monkeys during the terminal stage of the illness. Thus, it would appear that, as in humans, gastrointestinal bleeding does not play a significant role in the pathogenesis of the disease in monkeys. More consistent signs of bleeding were epistaxis, skin petechiae, and gingival hemorrhage.

Although the deposition of fibrin thrombi occurs during DIC, the severity of this finding is extremely variable [4,5]. It is more difficult to detect when DIC is of relatively short duration or when active fibrinolysis occurs. Detection of fibrin thrombi is further dependent on the fortuitous selection of appropriate tissue sections. Fibrin thrombus deposition was detected by microscopic examination of tissues in only one infected monkey in this study, but the presence of fibrin thrombi in the other infected monkeys is not precluded.

In conclusion, DIC is associated with the pathogenesis of DHF in M. mulatta. The coagulation abnormality appeared to be in the intrinsic coagulation pathway, and was indicated by the prolongation of the APTT. This prolongation could have been due to either decreased hepatic synthesis of clotting factors essential to the proper functioning of the intrinsic system or to activation of Hageman factor. The occurrence of early and progressive elevations of serum SDH in all of the infected monkeys was used as a measure of hepatocellular damage, which could have compromised the hepatic synthesis of clotting factors and other plasma proteins. The systemic arterial hypotension that occurred in infected monkeys may have been due to the combined effects of decreased hepatic synthesis of albumin with a concomitant decrease in the colloid osmotic pressure of plasma, and activation of the kinin system via activated Hageman factor.

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Table 1. Plasma fibrinogen levels in noninfected and Machupo virus-infected Macaca mulatta.

Day of study	Plasma fibrinogen ($\mu\text{g/ml} \pm \text{SE}$)		No. live/8 infected animals
	Noninfected animals	Virus-infected animals	
-4	258.5 ± 34.3	301.0 ± 34.1	8
-2	332.5 ± 20.2	363.3 ± 18.2	8
0	360.8 ± 26.6	354.3 ± 32.2	8
3	344.3 ± 28.8	325.6 ± 27.4	8
5	345.8 ± 25.6	371.5 ± 31.7	8
7	292.5 ± 40.2	422.8 ± 44.6	8
10	317.5 ± 10.4	576.8 ± 59.4	8
12	339.5 ± 18.2	527.5 ± 47.8	8
14	376.4 ± 2.6	516.0 ± 41.6	7
17	308.3 ± 15.0	623.0 ± 113.4	7
19	321.8 ± 29.4	431.3 ± 128.9	6
21	340.5 ± 15.3	264.0 ± 172.6	4

Figures

Figure 1. Mean rectal body temperature and arterial blood pressure in chair-restrained rhesus monkeys, measured before and after the inoculation of 1,000 pfu of Machupo virus ($n = 6$) or saline (controls, $n = 3$) on day 0. The onset and duration of viremia in virus-infected monkeys is indicated by the shaded area. Numbers in parentheses indicate the number of surviving infected monkeys tested that day. Standard errors of the mean are presented where values are significantly different from those of controls ($P < 0.05$).

Figure 2. Mean thrombocyte counts (top), activated partial thromboplastin times (middle), and fibrin split products (bottom) in cage-confined rhesus monkeys, measured before and after the inoculation of 1,000 pfu of Machupo virus ($n = 8$) or saline (controls, $n = 4$) on day 0. For fibrin split products, the numbers in parentheses indicate the number of infected monkeys with positive values on that day. The onset and duration of viremia in virus-infected monkeys is indicated by the shaded area. Standard errors of the means are presented where values are significantly different from those of controls ($P < 0.05$).

Figure 3. Mean concentrations of sorbitol dehydrogenase and albumin in sera of cage-confined rhesus monkeys, measured before and after the inoculation of 1,000 pfu of Machupo virus ($n = 8$) or saline (controls, $n = 4$) on day 0. The onset and duration of viremia in virus-infected monkeys is indicated by the shaded area. Standard errors of the means are presented where the values are significantly different from those of controls ($P < 0.05$).





